Supporting Information for:
Assessing the Effects of Silver Nanoparticles on Biological Nutrient Removal in
Bench-Scale Activated Sludge Sequencing Batch Reactors

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Ag Analytical Methods. Briefly, samples were diluted in an acid solution to digest stabilizing coatings and other compounds that could interfere with ICP-MS measurements. An Agilent 7700 inductively coupled plasma mass spectrometer (ICP-MS, Santa Clara, CA) was used to measure total and dissolved Ag. First, total Ag was measured by dissolving a sample of wastewater in 2% HNO₃ and 0.5% HCl concentrated acid for at least 24 h. Samples were separately taken to measure the amount of dissolved Ag in the wastewater. Dissolved Ag samples were defined as the fraction of Ag filtrate that can be passed through an Amicon® Ultra-4 Centrifugal Filter Unit (Millipore, Billerica, MA) and centrifuged at 7,000 rpm for 35 min. The filtrate was then acidified with nitric and hydrochloric acid using a method amended from Wang et al.¹.

DNA extraction and PCR Conditions. Biomass samples were taken periodically from the SBRs and micro-centrifuged for 1 min at 13,000 x g. Biomass samples were then immediately stored at -20°C until DNA extraction. Duplicate samples were extracted for

each treatment using the UltraClean® DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA). All DNA extractions were performed following the manufacturer's protocol. Following extraction, DNA was stored in elution buffer at -20°C until used for downstream processing. A ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to verify concentration and quality of DNA. PCR of the bacterial 16S SSU rRNA gene region was carried out by the methods described in Lukow et al. ² with small adjustment. 6 – carboxyfluorescein (6-FAM) was used to fluorescently label the forward primer (27F) and 1392R was used as the unlabeled reverse primer ³. First, 5 µL of template DNA was used for each amplification in a 100 µL reaction. The presence of the correct length PCR amplicons was confirmed by visualization on a 1% agarose gel containing 0.1% ethidium bromide. PCR amplicons were further purified by following the manufacturer's protocol in the Qiagen PCR Purification Kit (Qiagen, Hilden, Germany). Final PCR product concentrations and purity was verified as described above using the NanoDrop.

T-RFLP Analysis. Restriction enzyme digests were carried out by following the protocol described in Lukow et al. ². Ten units of *MspI* (New England Bio-labs Inc., Beverly, MA, USA) was added to 100 ng of purified PCR product and the manufacturer provided buffer solution. The mixture was incubated for 3 h at 37°C, followed by an enzymatic inactivation at 65°C for 15 min. Samples were stored on ice prior to the spin column filtration desalting step. Fragment analysis was carried out using an Applied Biosystems 3100 capillary sequencer (Foster City, CA) with POP6 polymer and ROX-labeled MapMarker 1000 size standards (BioVentures, Inc., Murfreesboro, TN). All analyses

were carried out at the Duke University DNA Analysis Facility (Durham, NC) following standard procedures. Applied Biosystems GeneScan v3.7.1 software (Foster City, CA) was used to interpret the raw data after electrophoresis. All samples were visually inspected for clean peaks and raw data was transferred into T-REX ⁴ in the resulting format: Dye/Peak, Sample File Name, Size, Height, Area, Data Point. T-REX aligned peaks and identified true peaks. Data analysis was performed in T-REX using the peak area feature, so that abundance and diversity could be examined. T-RFs smaller than 50 bp were not included in analysis, as to eliminate primer dimer fragments. The minimum peak height threshold was set at 50 relative fluorescent units. Data files were then imported into PAST statistical software to create Bray-Curtis principle coordinate graphs and cluster similarity (Hammer & Harper, D.A.T. 2006. Paleontological Data Analysis. Blackwell). For Bray-Curtis analysis, a transformation exponent of $c=2$ was selected. Eigenvalues were produced for coordinate plots. Diversity indices were calculated by transposing data into PAST and comparing taxonomic species with the Simpson 1-D index. The greater the resulting value, the more diverse the sample is in species.

Nitrite and Nitrate Concentrations in SBRs. Nitrate and nitrite concentrations were more variable than COD and ammonia removal trends during SBR treatment. An increase in nitrate was observed in the presence of Ag^+ and citrate AgNPs, which was attributed to the addition of nitrate from the Ag stock suspensions (approximately 6 ppm or 1mM contribution). This spike was not observed in GA AgNP treated SBRs. Nitrite concentrations were relatively steady throughout Ag dosing, with some slight increases in the Ag^+ treatment during the second pulse addition and the beginning of the continuous

spike. Since there was no surge in ammonia concentration during these time points, we speculate that this unusual effect was caused by a temporary shift in nitrite-oxidizing bacteria (NOB) metabolism causing ineffective nitrite removal.

Figure S1: Schematic and photos of SBRs used in this study. Picture (A) shows the set up of SBRs before addition of activated sludge. Picture (B) displays an overhead view of the SBRs during the aeration/mixing period. Graphic (C) shows a schematic of the SBRs.

Figure S2: Total Ag concentration during the 0.2 ppm spikes and continuous phase of Ag addition.

Figure S3: Total Ag concentration during the 2 ppm spikes and continuous phase of Ag addition.

Figure S4: Nitrate and Nitrite Concentrations during Ag Additions to SBRs

Table S1: Concentrations of Ag measured during the 14 d spike and the continuous phase for 0.2 and 2 ppm.

14 d Spike and Continuous Phase Average Ag Concentrations (ppb) - 0.2 ppm spikes								
SBR	1 min	std dev	1 day	std dev	14 days	std dev	Continuous Phase	std dev
Citrate AgNP	107.42	0.37	63.53	5.60	0.04	0.01	21.89	1.58
GA AgNP	123.61	5.61	8.89	0.10	0.03	0.00	11.79	0.74
Control	0.61	0.01	0.18	0.00	0.01	0.00	0.05	0.01
Ag as AgNO ₃	139.04	1.58	79.21	2.07	0.02	0.01	24.32	3.24
Citrate AgNP (dissolved)	0.19	0.03	0.21	0.00	0.01	0.00	0.01	0.00
GA AgNP (dissolved)	0.17	0.00	0.20	0.01	0.01	0.00	0.01	0.00
Control (dissolved)	0.16	0.00	0.21	0.01	0.01	0.00	0.00	0.00
Ag as AgNO ₃ (dissolved)	0.16	0.01	0.19	0.00	0.01	0.00	0.01	0.00

14 d Spike and Continuous Phase Average Ag Concentrations (ppm) - 2 ppm spikes								
SBR	1 min	std dev	1 day	std dev	14 days	std dev	Continuous Phase	std dev
Citrate AgNP	1.12	0.06	0.00	0.00	0.00	0.00	2.10	0.02
GA AgNP	0.73	0.15	0.00	0.00	0.00	0.00	0.55	0.03
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ag as AgNO ₃	0.77	0.26	0.00	0.00	0.00	0.00	1.07	0.12
Citrate AgNP (dissolved)	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
GA AgNP (dissolved)	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
Control (dissolved)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ag as AgNO ₃ (dissolved)	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00

Table S2: MINEQL speciation data of Ag in SWW

Name	Ag = 0.2 ppm		Ag = 2 ppm	
	Conc. (uM)	%Total	Conc. (uM)	%Total
Ag(+)	1.82E-07	9.8	1.84E-07	0
Ag(OH)2- (-1)	1.80E-17	0	1.82E-17	0
AgOH (aq)	1.61E-12	0	1.64E-12	0
AgNH3+ (+1)	2.24E-09	0	2.27E-09	0
Ag(NH3)2+ (+1)	1.10E-10	0	1.12E-10	0
AgH[EDTA] (-2)	9.14E-29	0	9.26E-29	0
AgCl2- (-1)	3.98E-08	2.2	3.93E-08	0
AgCl (aq)	3.63E-07	19.7	3.63E-07	2
AgCl3-2 (-2)	5.74E-11	0	5.59E-11	0
AgCl4-3 (-3)	2.44E-13	0	2.34E-13	0
Ag2MoO4 (aq)	5.87E-33	0	6.03E-33	0
AgSO4- (-1)	2.46E-10	0	2.49E-10	0
Ag[Acetate]	1.51E-08	0	1.53E-08	0
Ag[Acetate]2 (-1)	2.43E-10	0	2.46E-10	0
Ag[EDTA] (-3)	1.27E-28	0	1.29E-28	0
Ag[Glutamate] (-1)	1.97E-09	0	2.00E-09	0
Ag2[Glutamate]	4.22E-17	0	4.34E-17	0
Ag[Glutamate]2 (-3)	4.88E-12	0	4.95E-12	0
AgCitrate (-2)	7.32E-09	0	7.42E-09	0
CERARGYRITE	1.23E-06	66.8	1.79E-05	96.7

Table S3: AgNP physical characteristics

Type	Avg Radius	PDI	Primary Peak	ZP avg	stdev	TOC	stdev
Ag GA	32.31	0.474	44.78	-23	1.1	690	16
Ag Cit	15.49	0.536	29.86	-21	1.3	703.2	19.0

Table S4: Simpson 1-D diversity indices of Ag treated SBRs. Please refer to Table 2 for the labeling guide.

Diversity before Ag Addition			
Sample ID		Simpson 1-D	
CA0		0.954	
GA0		0.925	
C0		0.934	
AG0		0.931	

0.2 ppm Ag Additions		2 ppm Ag Additions	
Sample ID	Simpson 1-D	Sample ID	Simpson 1-D
CA14	0.708	CA14	0.597
GA14	0.252	GA14	0.066
C14	0.597	C14	0.721
AG14	0.846	AG14	0.049
CA6	0.841	CA6	0.701
GA6	0.867	GA6	0.895
C6	0.491	C6	0.251
AG6	0.926	AG6	0.049
CA2	0.721	CA2	0.877
GA2	0.585	GA2	0.077
C2	0.816	C2	0.049
AG2	0.411	AG2	0.086
CAcont	0.889	CAcont	0.064
GAcont	0.899	GAcont	0.022
Ccont	0.436	Ccont	0.055
AGcont	0.917	AGcont	0.846

Table S5: Bray-Curtis Similarity indices comparing the SBR bacterial community at the end of the 0.2 ppm continuous Ag addition and the community at the end of the 14 d 2 ppm spike. These values are used to identify shifts in the communities as the Ag concentration went up. A value closer to 0 indicates less similar communities.

	CAcont- 0.2 ppm	GAcont- 0.2 ppm	Ccont- 0.2 ppm	AGcont- 0.2 ppm	CA14-2 ppm	GA14-2 ppm	C14-2 ppm	AG14-2 ppm
CA14-2 ppm	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.00E+00	0.00E+00	2.33E-01	0.00E+00
GA14-2 ppm	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.00E+00	0.00E+00	9.75E-01
C14-2 ppm	1.22E-04	1.19E-04	0.00E+00	1.94E-04	2.33E-01	0.00E+00	1.00E+00	0.00E+00
AG14-2 ppm	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	9.75E-01	0.00E+00	1.00E+00
CAcont- 0.2 ppm	1.00E+00	2.74E-01	1.60E-01	3.06E-01	0.00E+00	0.00E+00	1.22E-04	0.00E+00
GAcont- 0.2 ppm	2.74E-01	1.00E+00	6.49E-02	3.23E-01	0.00E+00	0.00E+00	1.19E-04	0.00E+00
Ccont-0.2 ppm	1.60E-01	6.49E-02	1.00E+00	1.94E-01	0.00E+00	0.00E+00	0.00E+00	0.00E+00
AGcont- 0.2 ppm	3.06E-01	3.23E-01	1.94E-01	1.00E+00	0.00E+00	0.00E+00	1.94E-04	0.00E+00

References

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